YAC transgenics and the study of genetics and human disease

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Advances in yeast artificial chromosome (YAC) technologies over the past decade have enabled the precise identification and manipulation of large genomic regions (>100 kb) of DNA. Introduction of YACs into the mouse germline has now been accomplished through transfection of mouse embryonic stem cells as well as through pronuclear microinjection, allowing the efficient transfer of defined genomic loci into mice. YAC transgenics will have a profound impact on the development of transgenic mice as bioreactors and as models of human disease, and on the functional analysis of higher order genomic structure.

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Introduction

The stable introduction of new or altered genes into the germline of a mammal is one of the major technological advances in biology and has facilitated the study of gene function in development and disease. Experiments performed in the early 1980s demonstrated the feasibility and reproducibility of stably introducing DNA into single-cell mouse embryos through microinjection into the male pronucleus [1–5]. The study of such standard transgenic mice now represents a major strategy for the investigation of *in vivo* genetic and biological issues both in basic research and in industry.

It is well recognized that transgenes containing genomic DNA with introns and essential regulatory sequences are expressed more appropriately *in vivo* than cDNA-based constructs [6–9]. One substantial limitation of standard transgenic technology has been the inability to reproducibly introduce standard preparations of genomic fragments >40–50 kb, in part because of the mechanical shearing force encountered in a microinjection needle. In the past, this has severely hampered efforts to study genes, gene complexes, regulatory sequences and higher order genomic structure encompassing larger domains of DNA than can be cloned in plasmids and/or cosmids. Now, however, advances in yeast artificial chromosome (YAC), embryonic stem (ES) cell, and pronuclear microinjection technologies have overcome this obstacle.

YACs are cloning vectors with a DNA capacity of >2 Mb that are stably propagated in yeast [10]. They are extremely amenable to genetic manipulation by homologous recombination in yeast, permitting the efficient introduction of mutations, including deletions, insertions, and nucleotide substitutions, into precise locations within the YACs [11,12]. Homologous recombination also allows for the removal of unwanted DNA sequences present on YACs often introduced during the production of YAC libraries (chimeric YACs) as well as the generation of larger YACs from multiple smaller YACs. YACs have proven invaluable in mapping the genomes of numerous organisms and in the identification of disease genes.

ES cells are totipotent embryonic cells that can be cultured indefinitely *in vitro* [13] and can be genetically modified with precision, including the DNA sequence replacements and deletions utilized in gene 'knockouts' and even more subtle single base pair changes [14]. Modified ES cells are used subsequently to produce chimeric mice [15]. Transmission of ES cell DNA through the germline results in the production of transgenic mice containing the genetic modification.

Over the past two years, several publications have reported the introduction of YACs into the mouse germline (see Table 1). In this review of YAC transgenics, which is based on these reports, we will first compare and contrast the three different techniques that have been developed for introducing YACs into transgenic mice. We will then describe the specific applications of this technology with current examples and finally speculate as to the future direction of this work.

Abbreviations

Aβ—β-amyloid; **AD**—Alzheimer's disease; **APP**—amyloid precursor protein; **DS**—Down syndrome; **ES**—embryonic stem; ***neo***^{r}—neomycin resistance; **PFGE**—pulsed-field gel electrophoresis; **YAC**—yeast artificial chromosome.

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Table 1. Published reports of YAC transgenic mice (in chronological order).

Genes on YAC	Size of YAC	Method	Expression	References
Human *HPRT*	670 kb	Spheroplast fusion	Endogenous levels Tissue specific	[20••]
Mouse *tyrosinase*	250 kb	Microinjection	Endogenous levels	[26••]
Mouse *Col1a1*	150 kb	Lipofection	Endogenous levels	[18••]
Human *Ig* heavy chain	85 kb	Lipofection	Less than endogenous levels	[16•]
Human *Ig* light chain	300 kb	Spheroplast fusion	Expression in chimeric mice	[22]
Human *APP*	650 kb	Lipofection	Endogenous levels Tissue specific	[17••,19,37]
Human β-*globin* locus	150 and 248 kb	Microinjection	Endogenous levels Tissue specific	[24•,25]
Human *Ig* heavy and κ light chain	220 and 170 kb	Spheroplast fusion	High levels	[21••]

Techniques

Three different techniques have been utilized to introduce YACs into the mouse germline (represented diagrammatically in Fig. 1). Two of these techniques rely on the introduction of YACs into ES cells first and then into mice (Fig. 1a,b), and the third is a modification of standard pronuclear microinjection (Fig. 1c).

Lipofection

Lipofection requires the isolation of YACs from yeast cells, their purification away from the other yeast chromosomes by preparative pulsed-field gel electrophoresis (PFGE), the complexing of the YAC DNA with various lipid reagents, and their transfection into ES cells (Fig. 1a) [16•,17••,18••,19]. Selection for the presence of YACs in ES cells can be accomplished through the previous introduction of selectable cassettes, such as neomycin resistance (*neo*^{r}), into the YAC vector arm via homologous recombination in yeast [17••] or through co-lipofection of purified YAC DNA with an unlinked plasmid containing a *neo*^{r} cassette [16•,19]. Resulting ES colonies resistant to G418 (a neomycin analog) can be screened for the presence of integrated YAC DNA and for expression of previously identified genes present on the YAC. Lipofection with YACs containing a selectable cassette results in ~10% of the ES colonies retaining an integrated, intact and unrearranged copy of the YAC ([17••,18••]; BT Lamb, JD Gearhart, unpublished data), whereas co-lipofection with an unlinked selectable cassette results in 1% or fewer ES colonies containing the entire YAC [16•,19]. The majority of selected ES colonies in lipofection experiments contain fragments of the original YAC. YACs introduced via lipofection can integrate into ES cell DNA in single as well as multiple copies ([17••]; BT Lamb, JD Gearhart, unpublished data). The largest reported YAC introduced into transgenic mice by lipofection of ES cells is 650 kb [17••,19].

The lipofection of purified YACs into ES cells has its advantages; for example, no (or little) yeast DNA is transferred into ES cells, and the integrity and expression of genes on the YAC can be determined in ES cells prior to the production of transgenic mice. The disadvantages include the often tedious isolation of YAC DNA by PFGE, the fragmentation of YAC DNA during isolation and transfection, and the relatively long time required for the transmission of modified ES cells through the mouse germline.

Spheroplast fusion

An alternative approach for the introduction of YACs into ES cells involves the fusion of yeast spheroplasts containing a YAC with ES cells (Fig. 1b) [20••,21••,22]. A *neo*^{r} cassette (or other selectable cassette) is first introduced into the YAC by homologous recombination in the yeast. The cell wall of the yeast is removed subsequently by enzymatic digestion followed by a polyethylene glycol mediated fusion with the ES cells. Selection for the YAC results in ~40% of the ES colonies retaining a majority of the YAC as well as both YAC vector arms, either in single or multiple copies [23]. Not surprisingly, a majority of the ES colonies also contain varying amounts of yeast chromosomal DNA [20••,21••]. YACs up to 670 kb in length have been introduced into transgenic mice via spheroplast fusion [20••].

The technique of spheroplast fusion has a number of advantages: no YAC DNA isolation is necessary, a relatively high percentage of YACs are transferred to ES cells intact, and the integrity and expression of genes on the

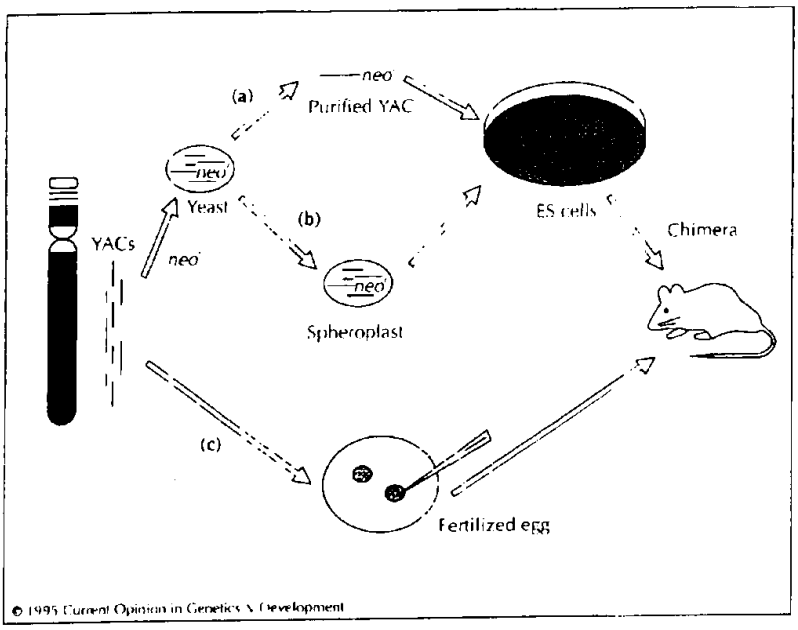

Fig. 1. Schematic representation of the three methods for the generation of YAC transgenic mice. **(a)** Lipofection involves the isolation of YACs containing a mammalian selectable cassette (*neo*) away from the other yeast chromosomes and subsequent introduction of purified YAC DNA into mouse ES cells via lipid-mediated transfection. **(b)** Spheroplast fusion relies on the introduction of YACs containing a selectable cassette into ES cells by enzymatic digestion of the yeast cell wall and fusion of the resulting spheroplasts with ES cells. ES cells containing YACs are subsequently introduced into host blastocyst-staged embryos, and germline transmission of ES cell DNA through the resulting chimeras generates YAC transgenic mice. **(c)** Microinjection involves the isolation of YACs away from the other yeast chromosomes, purification and concentration of the YAC DNA with modifications to limit the shearing of high molecular weight DNA in solution, and direct transfer of YAC DNA into the male pronucleus of mouse eggs.

YAC can be determined in ES cells prior to introduction into mice. The disadvantages include the simultaneous transfer of yeast DNA and the possible effects this may have on ES cells and transgenic mice and the relatively time-consuming transmission of modified ES cells through the mouse germline.

Microinjection

YACs can also be introduced into mice by direct microinjection into single-cell embryos (Fig. 1c) [24•,25,26••,27–29]. The preparation of YAC DNA suitable for microinjection has seen several major advances: purification by preparative PFGE, enzymatic digestion of agarose in the presence of high salt and/or polyamines to protect against the shearing of high molecular weight DNA in solution, and concentration (up to 1–5 ng μl^{-1}) by low-speed ultrafiltration [24•], dialysis with sucrose [25], or a second dimension electrophoresis [26••,28,29]. The concentrated YAC DNA is subsequently injected into the male pronucleus and resulting offspring are scored for the presence of various portions of the YAC. The efficiency of introducing YACs into mice via microinjection has been varied, but the most complete published data suggest that 1% or less of the oocytes injected lead to transgenic mice containing a majority of the YAC [28]. By contrast, standard pronuclear injection of smaller plasmid or cosmid DNA constructs results in ~10% or more of the oocytes injected yielding liveborn transgenic mice [7]. YAC transgenic mice produced by microinjection frequently contain fragmented YACs with single or multiple copies of DNAs integrated at either one or multiple sites in the genome [24•,26••]. Among published studies, the largest YAC transferred intact into mice by microinjection is 250 kb, although recent unpublished observations (BT Lamb, N Copeland, N Jenkins, JD Gearhart, unpublished data; E Rubin, personal communication, see Note added in proof) suggest that transfer of even larger YACs may be possible.

The technique of microinjection for introduction has a number of advantages: the production of transgenic mice via microinjection is relatively rapid, few yeast manipulations are required, no (or little) yeast DNA is transferred into mice, and any resulting mice can be tested immediately for the complementation of existing mouse mutants (see below). The disadvantages include the tedious and technically difficult isolation and concentration of intact YAC DNA, the shearing and fragmentation of YAC DNA during microinjection, and the possibility that larger YACs may be extremely difficult to microinject because of physical constraints of molarity of extremely large DNA molecules. As an example of the latter, purification of a 1000 kb YAC and concentration to 2 ng μl^{-1} would result in the optimal transfer of only 3.6 YACs in the standard microinjection volume of 2 pl, whereas microinjection of smaller DNAs generally results in the transfer of several hundred molecules.

Applications

The ability to transfer large cloned chromosomal regions of DNA into mice provides a unique opportunity to investigate many basic issues in genetic and biological research. Such applications include the introduction of large genes and gene complexes for the development of

transgenic mice as bioreactors, the generation of unique transgenic models of human genetic diseases, and the functional analysis of higher order genomic structure. The published reports of YAC transgenic mice and their applications are summarized in Table 1 in chronological order.

Bioreactors

Transgenic mice are a unique vehicle for the expression and purification of foreign proteins. Many genetic loci of interest are extremely large and complex, however, and would be impossible to introduce intact by standard pronuclear microinjection. The *immunoglobulin* (*Ig*) heavy and light chain genes, for example, span >2 Mb each, with a complex arrangement of numerous V, D, and J sequences that encode the variable domains, as well as the C sequences that encode constant domains of Ig molecules [30]. It is the unique recombination of these sequences in mature B cells that accounts for the unique diversity of the antibody response. Clearly, the ability to introduce the entire human heavy and light chain *Ig* loci into transgenic mice with the concomitant expression of diverse antigen-specific human antibodies would be desirable for human therapeutic purposes.

Several different groups have introduced YACs containing genomic portions of both the *Ig* heavy and light chain genes into transgenic mice by lipofection [16•] and spheroplast fusion [21••,22]. In all of these instances, the human genes were recombined in the transgenic mice and appreciable levels of human Ig products were expressed. When both heavy and light chain genes were introduced, the mice expressed both membrane-bound and fully secreted human antibodies [21••]. The introduction of the *Ig* YAC transgenes into mice in which both the endogenous heavy and light chain genes were inactivated by homologous recombination resulted in the production of transgenic mice with an antigen-specific human antibody response [21••]. Introduction of even larger YACs containing the complete human heavy and light chain *Ig* loci into transgenic mice should generate a complete human antibody repertoire in mice. A similar transgenic strategy may be employed for other large complex mammalian loci, resulting in the production of valuable foreign proteins in the mouse.

Models of human disease

The ability to introduce large cloned genomic regions into transgenic mice has profound implications for the development of animal models of human disease. This is particularly obvious for the production of animal models of DNA duplications which result in human diseases, such as Charcot–Marie–Tooth disease type 1A [31], as well as chromosome dosage imbalance, such as human trisomies 13, 18, and 21. Trisomy 21, or Down syndrome (DS), is the most common aneuploidy among liveborn humans and is one of the leading known genetic causes of mental retardation [32]. DS individuals exhibit any number of phenotypic features in addition to mental retardation, including congenital heart disease, hand anomalies, a characteristic facial appearance, as well as Alzheimer's disease (AD) neuropathology. Considerable research effort has been focused on accurately defining the phenotypic characteristics of DS and on identifying DNA sequences on human chromosome 21 that contribute to the features of the syndrome.

Several groups have introduced discrete genomic regions of chromosome 21 cloned in YACs into transgenic mice in attempts to correlate dosage imbalance of discrete genes with specific DS features ([17••,19]; E Rubin, personal communication, see Note added in proof). In one example, to test the hypothesis that dosage imbalance and overexpression for the *amyloid precursor protein* (*APP*) gene on chromosome 21 may lead to phenotypic and neuropathologic characteristics of AD and/or DS in an animal model, a 650 kb human YAC containing the 400 kb *APP* gene was introduced into transgenic mice by lipofection [17••,19]. APP is an integral membrane protein which gives rise to the 39–42 amino acid β-amyloid (Aβ) peptide found in dense fibrillar deposits in the brains of aged individuals with AD and DS [33–36]. Consistent with a dosage imbalance model, the *APP* YAC transgenic mice produce high levels of human APP mRNA and protein that mirrors the expression of endogenous mouse APP products in both brain and peripheral tissues [17••,19,37]. In addition, primary neurons from the YAC transgenic mice produce human Aβ peptide at levels similar to mouse Aβ (BT Lamb, JD Gearhart, Roundtable on Transgenic Models of Alzheimer's Disease, 4th International Conference on Alzheimer's Disease and Related Disorders, Minneapolis MN, August 1994). Although these animals have not shown evidence of amyloid deposition or other obvious neuropathology consistent with AD and DS (BT Lamb, JD Gearhart, unpublished data), they are continuing to be examined for more subtle abnormalities. As described below, perhaps additional genetic and/or physical insults will be required to assess the role of *APP* in the etiology of AD and DS. In addition to *APP*, other specific genomic regions cloned in YACs from chromosome 21, including those encompassing the *SOD1*, *GART*, and *AML-1* genes, are also being introduced in transgenic mice in an effort to examine the effect of dosage imbalance for certain genes both independently and in combination (BT Lamb, JD Gearhart, unpublished data).

YAC transgenic technology will also prove useful in understanding the role of large complex genes in the etiology of numerous dominant genetic defects. Again, animal models of AD will serve as an example. The discovery of specific mutations in *APP* associated with the presence of disease and Aβ deposition in some cases of early-onset familial AD [38–41] provides compelling evidence for the involvement of APP in the pathogenesis of AD. As mentioned above, *APP* is an extremely large gene comprising 18 exons with three major alternatively spliced RNAs that are expressed in a tissue- and devel-

opmental stage specific manner. Thus, YAC transgenic animals which contain the complete genomic sequence of *APP* will allow the stringent testing of the roles of the familial AD mutations on amyloidogenesis in an animal model. Towards this end, specific familial AD point mutations have been introduced into the 650 kb *APP* YAC by homologous recombination in yeast via a two-step gene replacement ([42]; BT Lamb, LM Call, HH Slunt, SS Sisodia, KF Boese, JD Gearhart, abstract 47, 4th International Conference on Alzheimer's Disease and Related Disorders, Minneapolis MN, August 1994). YAC transgenesis may prove critical for defining pathogenesis of other dominant genetic disorders including Huntington's disease [43] and Marfan syndrome [44].

Analysis of mammalian genomes

The various mammalian genome initiatives which aim to map and sequence entire genomes will probably provide substantial information about the location and organization of most genetic loci. They will not, however, provide substantial insight into functional aspects of genes as well as higher order genomic structure. YAC transgenics can potentially help to bridge the gap between *in vitro* genome structure and *in vivo* genome function. Research areas likely to be impacted include gene identification through complementation of existing mutations, the regulation of gene complexes and far-acting regulatory sequences, and higher order genomic structure such as X-chromosome inactivation and imprinting.

Mouse geneticists have long desired powerful techniques to assist in the physical mapping and molecular characterization of mouse mutations. YAC transgenics should enable the introduction of large genomic segments into mice for the functional complementation of existing recessive mutations. YACs that demonstrate *in vivo* complementation can then be fragmented or specific sequences deleted through homologous recombination in yeast, followed by re-introduction into transgenic mice to further define the DNA sequences responsible. In addition, cDNA coding sequences on YACs can be identified readily through well characterized techniques such as direct selection [45] and exon trapping [46]. The combination of these powerful approaches should hasten the identification of the precise genetic deficits responsible for numerous mouse mutants.

The strongest evidence that such a YAC transgenic approach is feasible comes from a pilot study performed on the mouse *albino* mutation, a recessive mutation that results in a lack of pigmentation in melanocytes and retinal pigment epithelium as well as abnormal neuronal pathways projecting from the retina. The well characterized *albino* genetic defect involves mutations in the *tyrosinase* gene [47–49], the product of which is a key enzyme in the synthesis of melanin. In an effort to complement the *albino* mutation, Schütz and colleagues [26••,29] introduced a 250 kb YAC containing the 80 kb mouse *tyrosinase* gene into transgenic mice by microinjection. Transgene-encoded mRNAs reached levels comparable to that of the endogenous *tyrosinase* gene in a copy number dependent manner, and the YAC transgene corrected not only the lack of pigmentation in melanocytes, but also the abnormal retinal chiasmatic pathways ([26••]; G Schütz, personal communication). A similar approach may prove invaluable in the actual identification of the molecular defects responsible for other mouse mutants.

Studies of gene complexes and far-acting regulatory sequences will also be profoundly affected by the advent of YAC transgenics. Towards this end, two different groups have utilized YACs to study the expression of human β-*globin* genes in mice. The β-*globin* locus contains five functional genes that are arranged on the chromosome in the order in which they are expressed during development [50]. Expression of the β-*globin* genes is regulated precisely in a erythroid- and developmental stage specific manner. Various regulatory sequences spanning >150 kb have been implicated in this coordinated expression pattern, and thus *in vivo* studies of the entire locus have proven difficult. Introduction of a 248 or 150 kb YAC containing the entire β-*globin* locus into transgenic mice by microinjection resulted in the proper temporal and tissue-specific expression of the five β-*globin* genes [24•,25]. Utilizing homologous recombination in yeast, it should now be possible to directly test the effect of specific *cis*-acting sequences on the entire β-*globin* locus. Similar studies can be envisioned for the regulation of large genes, as well as other gene complexes such as the *homeobox* and *major histocompatibility* loci.

Finally, YAC transgenesis will also enable a more complete analysis of higher order mammalian genomic structure and function, including insights into X-chromosome inactivation, imprinting, chromosomal domains, origins of replication, and centromeres. X-chromosome inactivation, for example, is a complex process that involves the random transcriptional silencing of one of the X-chromosomes in mammalian XX females [51]. Studies in both mouse and man have demonstrated that X inactivation is dependent on an X-inactivation center, through an ill-defined chromosome-counting mechanism. One gene that has been implicated in playing a role in X inactivation is *XIST*. The human *XIST* gene encodes a 17 kb transcript that is expressed exclusively from the inactive X-chromosome [52]. Although some evidence exists that *XIST* is involved in the initiation of X-chromosome inactivation, it is (as yet) unclear whether *XIST* is also involved in the spread or maintenance of X inactivation [51]. To more clearly dissect the role of *XIST* as well as other genes in X inactivation, it should be possible to introduce large regions of the X-inactivation complex cloned in YACs first into ES cells and then possibly into transgenic mice. Critical DNA sequences can then be defined and analyzed further by specifically manipulating the YACs by homologous recombination in yeast before transfer into ES cells. Similar strategies may prove critical in correlating the structural information obtained from the mammalian genome initiatives to functional analysis in the whole organism.

Note added in proof

Studies referred to in the text as E Rubin, personal communication involving the production of YAC transgenic mice containing the *apolipoprotein(a)* gene and YACs from human chromosome 21 via pronuclear microinjection have now been published [53] or accepted as in press [54], respectively.

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